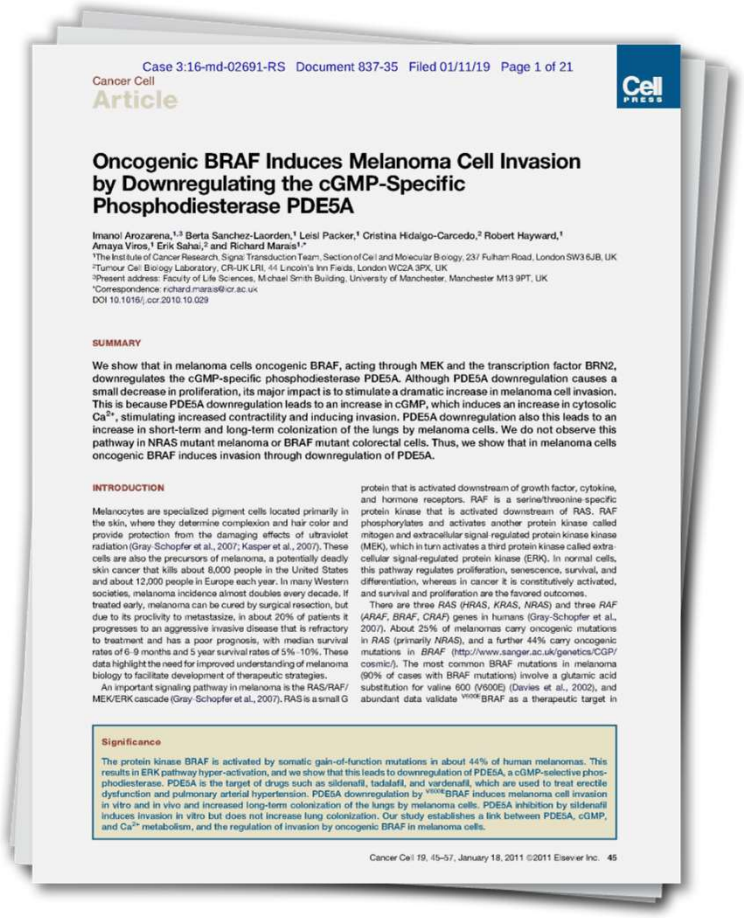
Direct Examination of Dr. Richard Marais

VIAGRA DAUBERT HEARING

Why Plaintiffs' Experts Unreliably Interpret Arozarena et al.



Cancer Cell
Article

Cell PRESS

Oncogenic BRAF Induces Melanoma Cell Invasion by Downregulating the cGMP-Specific Phosphodiesterase PDE5A

Imanol Arozarena,[1,3] Berta Sanchez-Laorden,[1] Leisl Packer,[1] Cristina Hidalgo-Carcedo,[2] Robert Hayward,[1] Amaya Viros,[1] Erik Sahai,[2] and Richard Marais[1,*]
[1]The Institute of Cancer Research, Signal Transduction Team, Section of Cell and Molecular Biology, 237 Fulham Road, London SW3 6JB, UK
[2]Tumour Cell Biology Laboratory, CR-UK LRI, 44 Lincoln's Inn Fields, London WC2A 3PX, UK
[3]Present address: Faculty of Life Sciences, Michael Smith Building, University of Manchester, Manchester M13 9PT, UK
*Correspondence: richard.marais@icr.ac.uk
DOI 10.1016/j.ccr.2010.10.029

SUMMARY

We show that in melanoma cells oncogenic BRAF, acting through MEK and the transcription factor BRN2, downregulates the cGMP-specific phosphodiesterase PDE5A. Although PDE5A downregulation causes a small decrease in proliferation, its major impact is to stimulate a dramatic increase in melanoma cell invasion. This is because PDE5A downregulation leads to an increase in cGMP, which induces an increase in cytosolic Ca^{2+}, stimulating increased contractility and inducing invasion. PDE5A downregulation also this leads to an increase in short-term and long-term colonization of the lungs by melanoma cells. We do not observe this pathway in NRAS mutant melanoma or BRAF mutant colorectal cells. Thus, we show that in melanoma cells oncogenic BRAF induces invasion through downregulation of PDE5A.

INTRODUCTION

Melanocytes are specialized pigment cells located primarily in the skin, where they determine complexion and hair color and provide protection from the damaging effects of ultraviolet radiation (Gray-Schopfer et al., 2007; Kasper et al., 2007). These cells are also the precursors of melanoma, a potentially deadly skin cancer that kills about 8,000 people in the United States and about 12,000 people in Europe each year. In many Western societies, melanoma incidence almost doubles every decade. If treated early, melanoma can be cured by surgical resection, but due to its proclivity to metastasize, in about 20% of patients it progresses to an aggressive invasive disease that is refractory to treatment and has a poor prognosis, with median survival rates of 6–9 months and 5 year survival rates of 5%–10%. These data highlight the need for improved understanding of melanoma biology to facilitate development of therapeutic strategies.

An important signaling pathway in melanoma is the RAS/RAF/MEK/ERK cascade (Gray-Schopfer et al., 2007). RAS is a small G protein that is activated downstream of growth factor, cytokine, and hormone receptors. RAF is a serine/threonine-specific protein kinase that is activated downstream of RAS. RAF phosphorylates and activates another protein kinase called mitogen and extracellular signal-regulated protein kinase kinase (MEK), which in turn activates a third protein kinase called extracellular signal-regulated protein kinase (ERK). In normal cells, this pathway regulates proliferation, senescence, survival, and differentiation, whereas in cancer it is constitutively activated, and survival and proliferation are the favored outcomes.

There are three *RAS* (*HRAS*, *KRAS*, *NRAS*) and three *RAF* (*ARAF*, *BRAF*, *CRAF*) genes in humans (Gray-Schopfer et al., 2007). About 25% of melanomas carry oncogenic mutations in *RAS* (primarily *NRAS*), and a further 44% carry oncogenic mutations in *BRAF* (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The most common BRAF mutations in melanoma (90% of cases with BRAF mutations) involve a glutamic acid substitution for valine 600 (V600E) (Davies et al., 2002), and abundant data validate ^{V600E}BRAF as a therapeutic target in

Significance

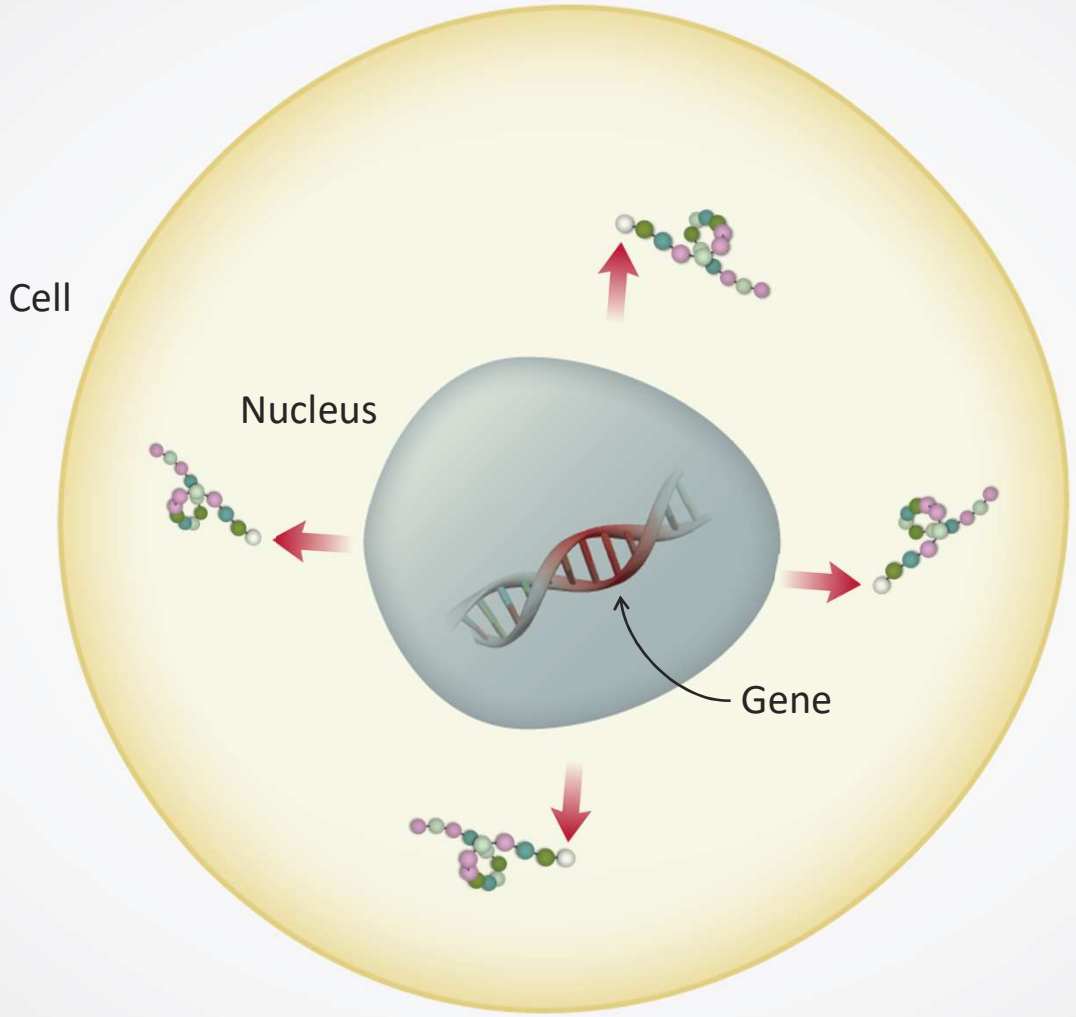
The protein kinase BRAF is activated by somatic gain-of-function mutations in about 44% of human melanomas. This results in ERK pathway hyper-activation, and we show that this leads to downregulation of PDE5A, a cGMP-selective phosphodiesterase. PDE5A is the target of drugs such as sildenafil, tadalafil, and vardenafil, which are used to treat erectile dysfunction and pulmonary arterial hypertension. PDE5A downregulation by ^{V600E}BRAF induces melanoma cell invasion in vitro and in vivo and increased long-term colonization of the lungs by melanoma cells. PDE5A inhibition by sildenafil induces invasion in vitro but does not increase lung colonization. Our study establishes a link between PDE5A, cGMP, and Ca^{2+} metabolism, and the regulation of invasion by oncogenic BRAF in melanoma cells.

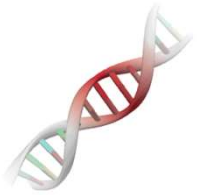
Plaintiffs' experts improperly:

1. Equate genetic manipulation with inhibition by drugs
2. Present a single *in vitro* result out of context
3. Discount our *in vivo* result
4. Mischaracterize our conclusions

Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011.

How Genes and Proteins Interact In Cells

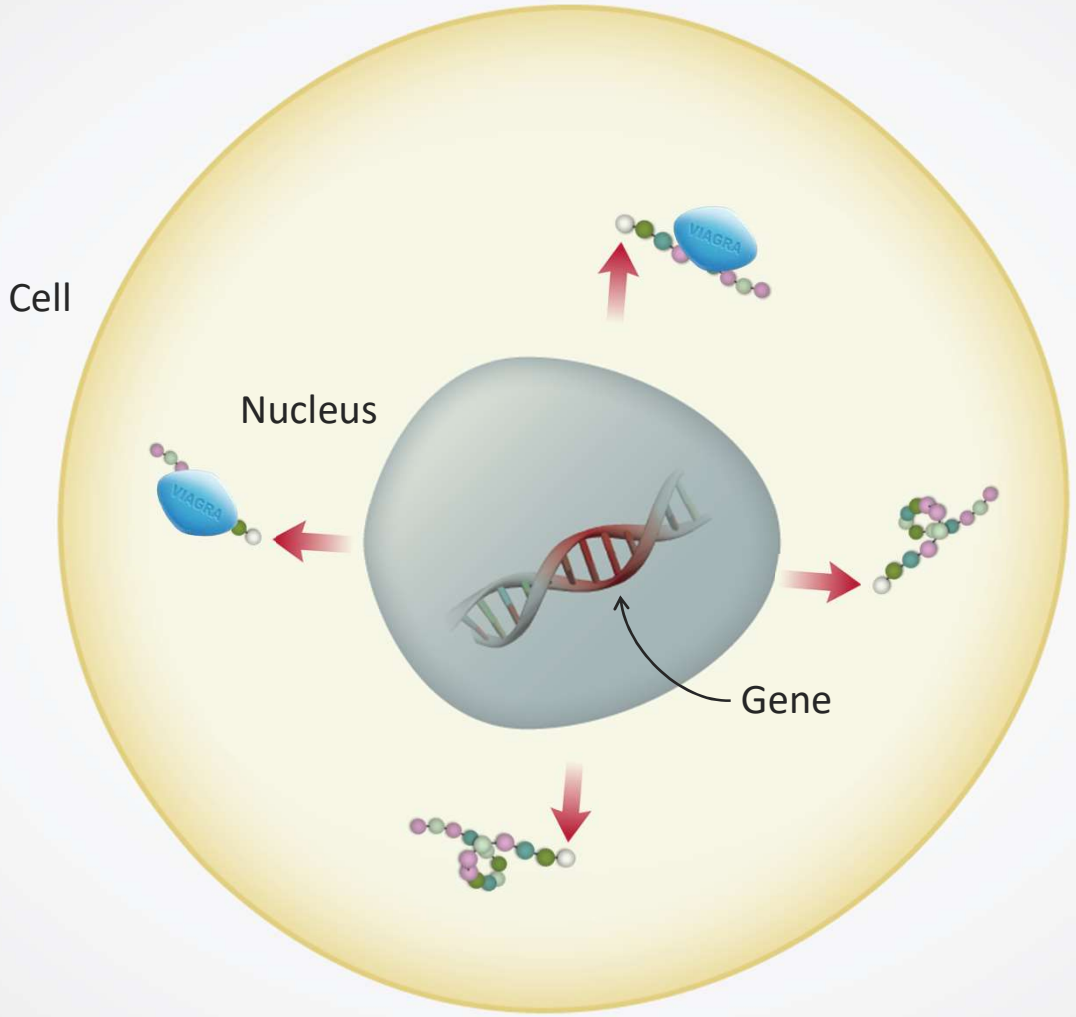




Genes are blueprints for the cell

Proteins are produced

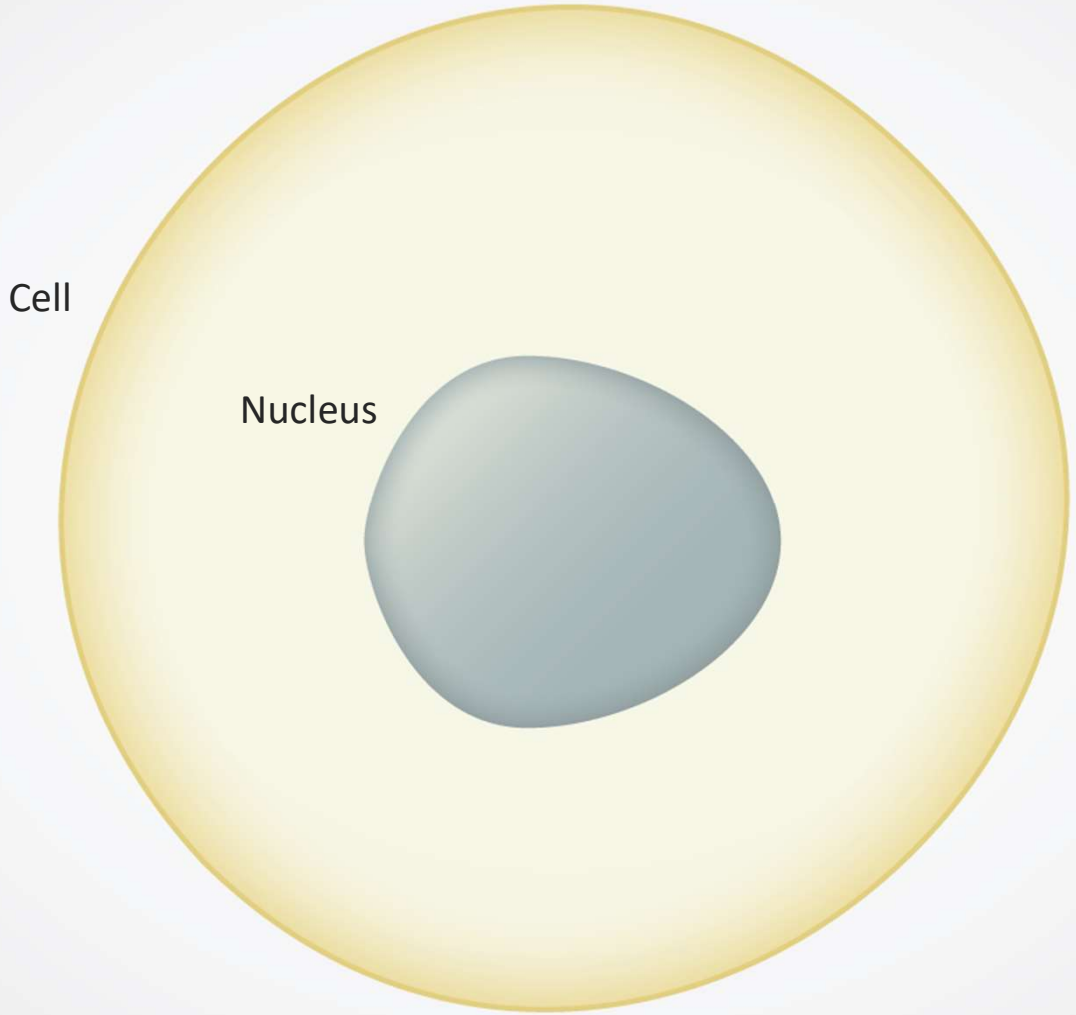
How Genes and Proteins Interact In Cells



Genes are blueprints for the cell

Proteins are produced

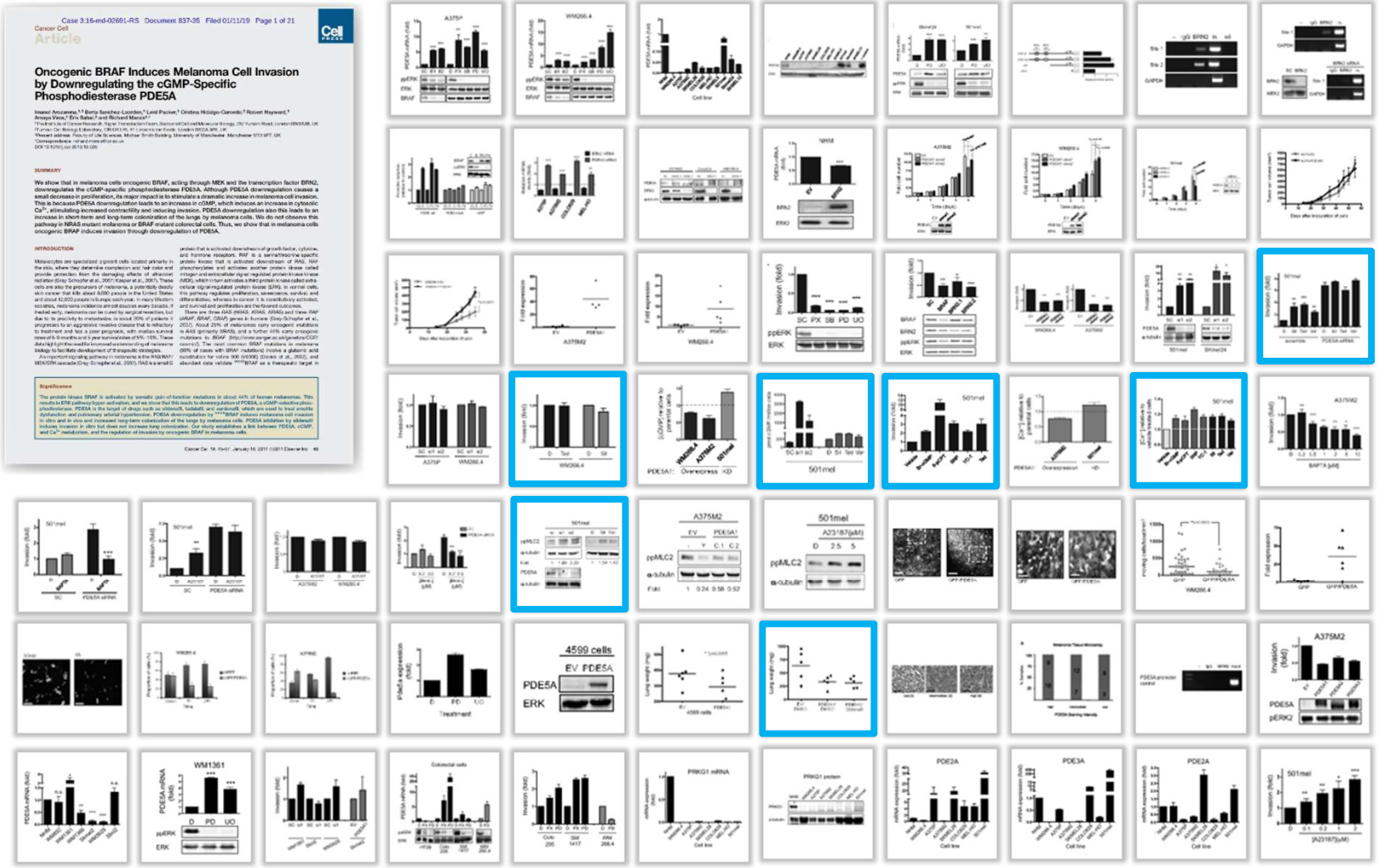
How Genes and Proteins Interact In Cells



Genes are blueprints for the cell

Proteins are produced

Only 7 of 65 Figures in Arozarena et al. Involve PDE5 Inhibitors



Case 3:16-md-02691-RS Document 837-35 Filed 01/11/19 Page 1 of 21

Cancer Cell

Article

Cell PRESS

Oncogenic BRAF Induces Melanoma Cell Invasion by Downregulating the cGMP-Specific Phosphodiesterase PDE5A

Imanol Arozarena,[1,3] Berta Sanchez-Laorden,[1] Loral Packer,[1] Cristina Hidalgo-Carcedo,[1] Robert Hayward,[1] Amaya Viros,[1] Eric Sahai,[2] and Richard Marais[1,*]

[1] The Institute of Cancer Research: Royal Cancer Hospital, Translational Cell and Molecular Biology, 237 Fulham Road, London SW3 6JB, UK

[2] Tumour Cell Biology Laboratory, CR-UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

[3] Present address: Faculty of Life Sciences, Michael Smith Building, University of Manchester, Manchester M13 9PT, UK

*Correspondence: richard.marais@icr.ac.uk

DOI 10.1016/j.ccr.2010.10.029

SUMMARY

We show that in melanoma cells oncogenic BRAF, acting through MEK and the transcription factor BRN2, downregulates the cGMP-specific phosphodiesterase PDE5A. Although PDE5A downregulation causes a small decrease in proliferation, its major impact is to stimulate a dramatic increase in melanoma cell invasion. This is because PDE5A downregulation leads to an increase in cGMP, which induces an increase in cytosolic Ca^{2+}, stimulating increased contractility and reducing invasion. PDE5A downregulation also this leads to an increase in short-term and long-term colonization of the lungs by melanoma cells. We do not observe this pathway in NRAS mutant melanoma or BRAF mutant colorectal cells. Thus, we show that in melanoma cells oncogenic BRAF induces invasion through downregulation of PDE5A.

INTRODUCTION

Melanocytes are specialized pigment cells located primarily in the skin, where they determine complexion and hair color and provide protection from the damaging effects of ultraviolet radiation (Gray-Schopfer et al., 2007; Kupper et al., 2007). These cells are also the precursors of melanoma, a potentially deadly skin cancer that kills about 8,000 people in the United States and about 12,000 people in Europe each year, in many Western societies, melanoma incidence almost doubles every decade. If treated early, melanoma can be cured by surgical resection, but due to its proclivity to metastasize, in about 20% of patients it progresses to an aggressive invasive disease that is refractory to treatment and has a poor prognosis, with median survival rates of 6–9 months and 5 year survival rates of 5%–10%. These data highlight the need for improved understanding of melanoma biology to facilitate development of therapeutic strategies.

An important signaling pathway in melanoma is the RAS/RAF/MEK/ERK cascade (Gray-Schopfer et al., 2007). RAS is a small G protein that is activated downstream of growth factor, cytokine, and hormone receptors. RAF is a serine/threonine specific protein kinase that is activated downstream of RAS. RAF phosphorylates and activates another protein kinase called mitogen-and-extracellular signal-regulated protein kinase kinase (MEK), which in turn activates a third protein kinase called extracellular signal-regulated protein kinase (ERK). In normal cells, this pathway regulates proliferation, senescence, survival, and differentiation, whereas in cancer it is constitutively activated, and survival and proliferation are the favored outcomes.

There are three RAS (HRAS, KRAS, NRAS) and three RAF (ARAF, BRAF, CRAF) genes in humans (Gray-Schopfer et al., 2007). About 25% of melanomas carry oncogenic mutations in RAS (primarily NRAS), and a further 66% carry oncogenic mutations in BRAF (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The most common BRAF mutations in melanoma (99% of cases with BRAF mutations) involve a glutamic acid substitution for valine 600 (V600E) (Davies et al., 2002), and abundant data validate ^{V600E}BRAF as a therapeutic target in

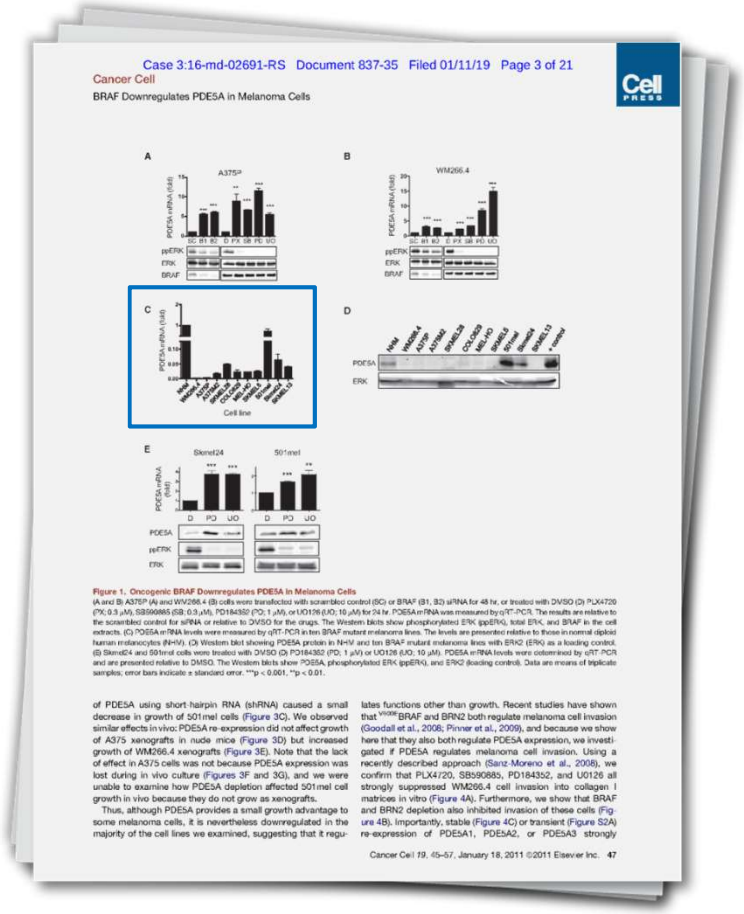
Significance

The protein kinase BRAF is activated by somatic gain-of-function mutations in about 44% of human melanomas. This results in ERK pathway hyper-activation, and we show that this leads to downregulation of PDE5A, a cGMP-selective phosphodiesterase. PDE5A is the target of drugs such as sildenafil, tadalafil, and vardenafil, which are used to treat erectile dysfunction and pulmonary arterial hypertension. PDE5A downregulation by ^{V600E}BRAF induces melanoma cell invasion in vitro and in vivo and increased long-term colonization of the lungs by melanoma cells. PDE5A inhibition by sildenafil induces invasion in vitro but does not increase long colonization. Our study establishes a link between PDE5A, cGMP, and Ca^{2+} metabolism, and the regulation of invasion by oncogenic BRAF in melanoma cells.

Cancer Cell 19, 45–57, January 18, 2011 ©2011 Elsevier Inc. 45

Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011 (highlights are of Figures 4E, 4G, 5B, 5C, 5E, 6B, 7K).

501mel Has Very High PDE5A Expression, Unlike Other Cell Lines



Cancer Cell

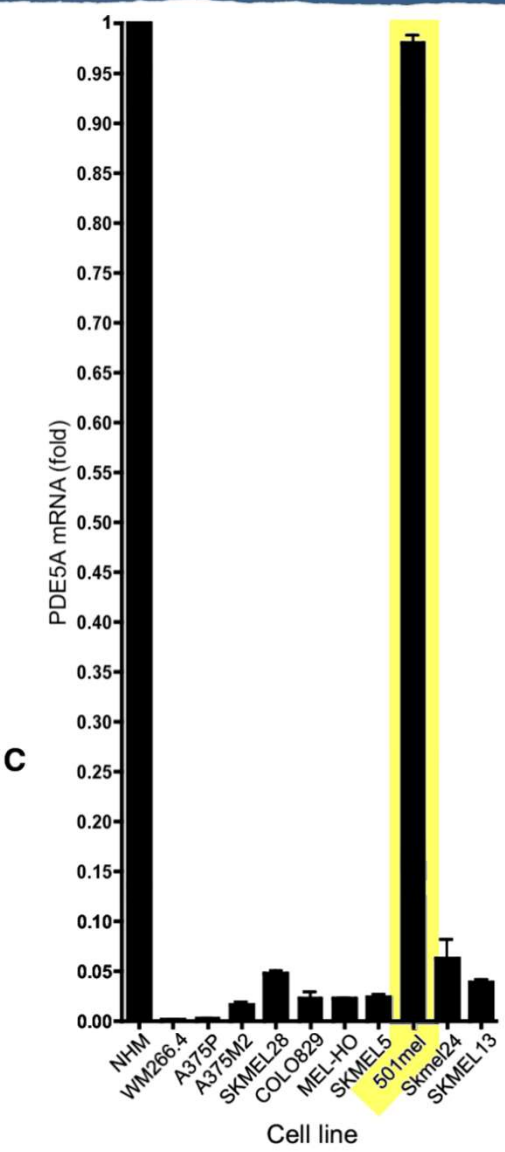
BRAF Downregulates PDE5A in Melanoma Cells

Figure 1. Oncogenic BRAF Downregulates PDE5A in Melanoma Cells

(A and B) A375P (A) and WM266.4 (B) cells were transfected with scrambled control (SC) or BRAF (B1, B2) siRNA for 48 hr, or treated with DMSO (D) PLX4720 (PX; 0.3 μM), SB590885 (SB; 0.3 μM), PD184352 (PD; 1 μM), or UO126 (UO; 10 μM) for 24 hr. PDE5A mRNA was measured by qRT-PCR. The results are relative to the scrambled control for siRNA or relative to DMSO for the drugs. The Western blots show phosphorylated ERK (ppERK), total ERK, and BRAF in the cell extracts. (C) PDE5A mRNA levels were measured by qRT-PCR in ten BRAF mutant melanoma lines. The levels are presented relative to those in normal diploid human melanocytes (NHM). (D) Western blot showing PDE5A protein in NHM and ten BRAF mutant melanoma lines with ERK2 (ERK) as a loading control. (E) Skmel24 and 501mel cells were treated with DMSO (D) PD184352 (PD; 1 μM) or UO126 (UO; 10 μM). PDE5A mRNA levels were determined by qRT-PCR and are presented relative to DMSO. The Western blots show PDE5A, phosphorylated ERK (ppERK), and ERK2 (loading control). Data are means of triplicate samples; error bars indicate ± standard error. $^{***}p < 0.001$, $^{**}p < 0.01$.

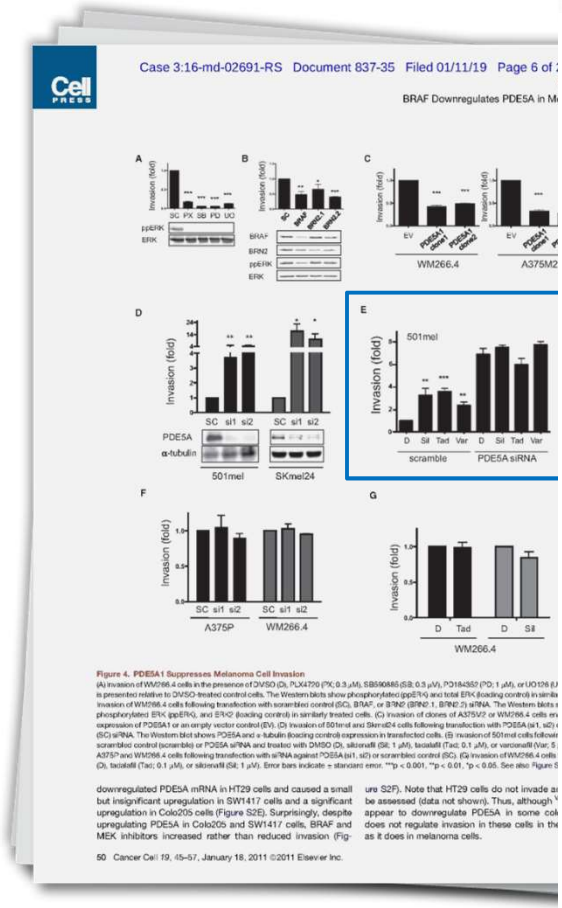
of PDE5A using short-hairpin RNA (shRNA) caused a small decrease in growth of 501mel cells (Figure 3C). We observed similar effects in vivo: PDE5A re-expression did not affect growth of A375 xenografts in nude mice (Figure 3D) but increased growth of WM266.4 xenografts (Figure 3E). Note that the lack of effect in A375 cells was not because PDE5A expression was lost during in vivo culture (Figures 3F and 3G), and we were unable to examine how PDE5A depletion affected 501mel cell growth in vivo because they do not grow as xenografts.

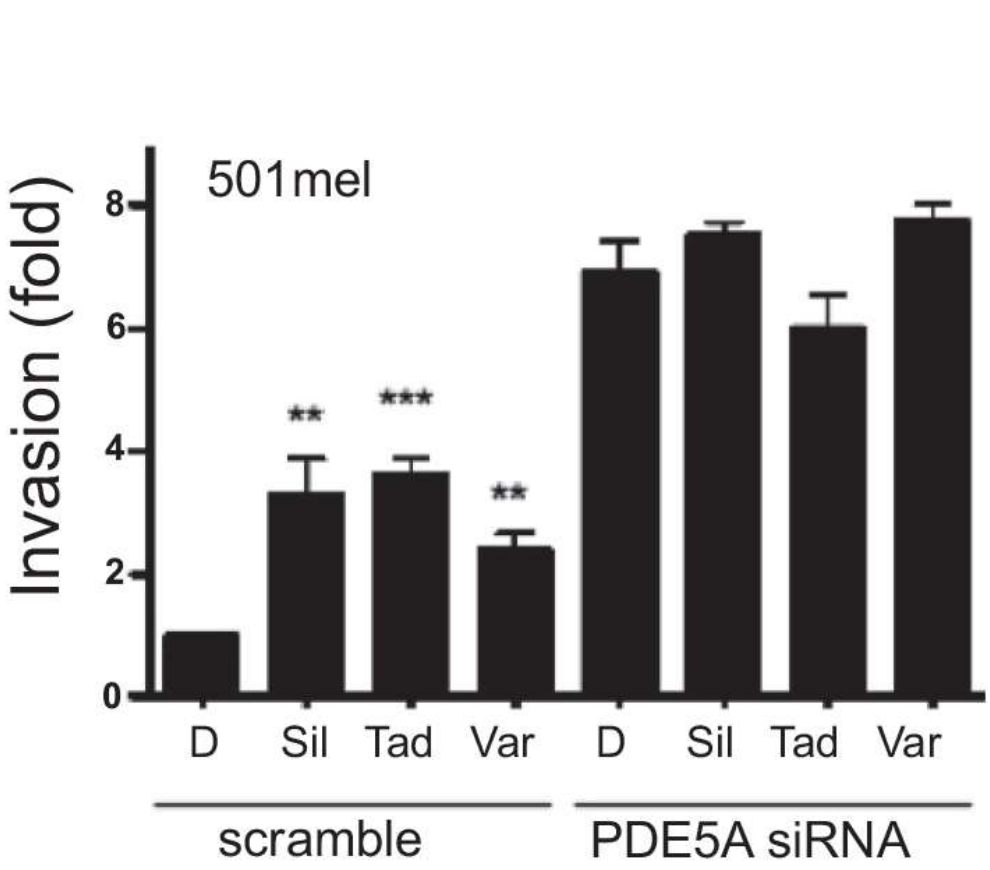
Thus, although PDE5A provides a small growth advantage to some melanoma cells, it is nevertheless downregulated in the majority of the cell lines we examined, suggesting that it regulates functions other than growth. Recent studies have shown that ^{V600E}BRAF and BRN2 both regulate melanoma cell invasion (Goodall et al., 2008; Pinner et al., 2009), and because we show here that they also both regulate PDE5A expression, we investigated if PDE5A regulates melanoma cell invasion. Using a recently described approach (Sanz-Moreno et al., 2008), we confirm that PLX4720, SB590885, PD184352, and U0126 all strongly suppressed WM266.4 cell invasion into collagen I matrices in vitro (Figure 4A). Furthermore, we show that BRAF and BRN2 depletion also inhibited invasion of these cells (Figure 4B). Importantly, stable (Figure 4C) or transient (Figure S2A) re-expression of PDE5A1, PDE5A2, or PDE5A3 strongly



Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011, at p. 47, Fig. 1C.

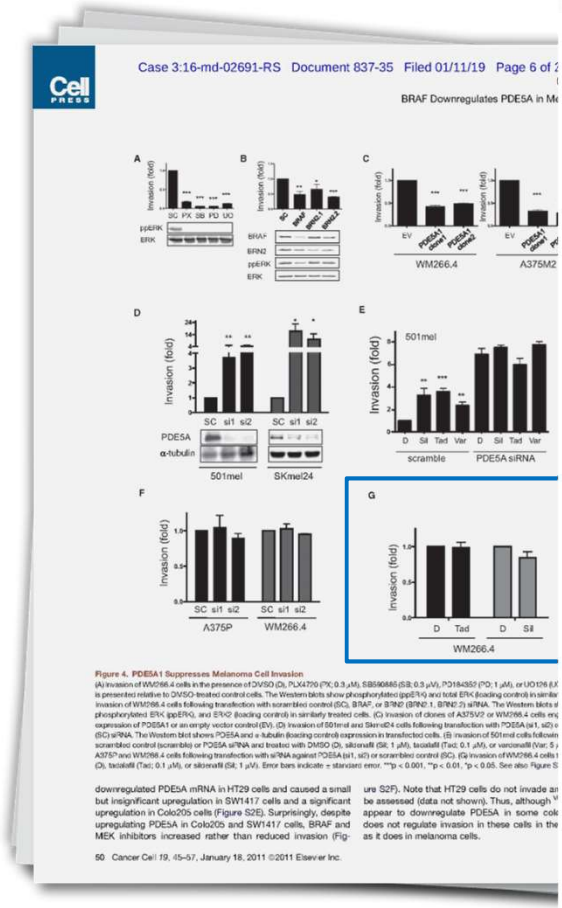
In Vitro: Increased Collagen Movement in 501mel (Very High PDE5A Expression)

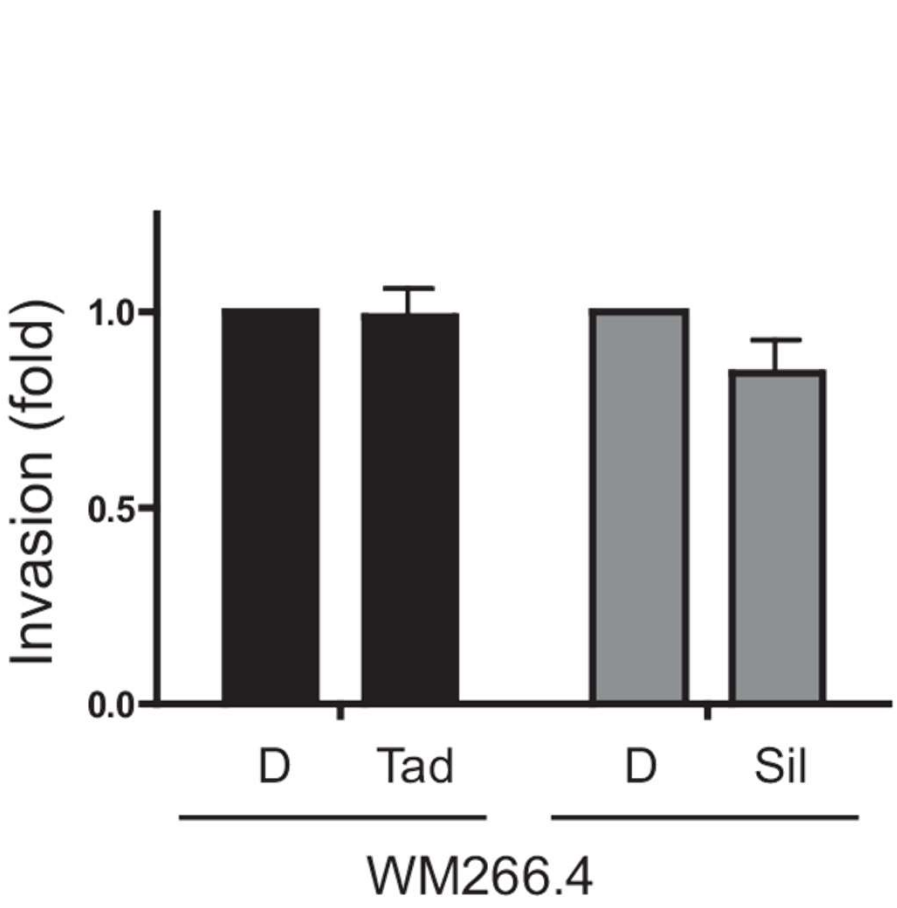




Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011, at p. 50, Fig. 4E.

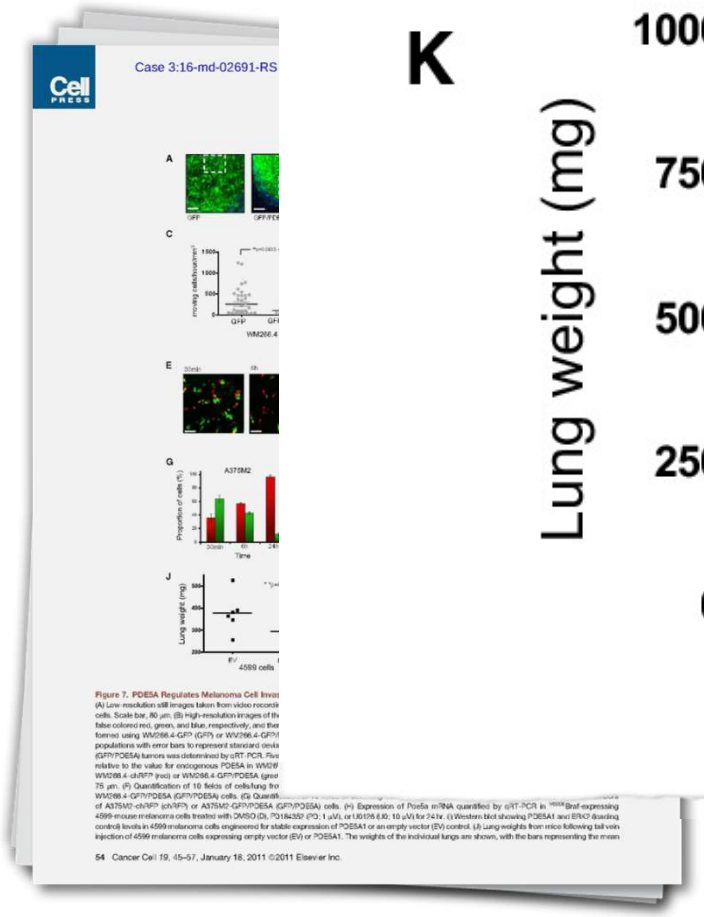
In Vitro: No Increased Collagen Movement in WM266.4 (Low PDE5A Expression)

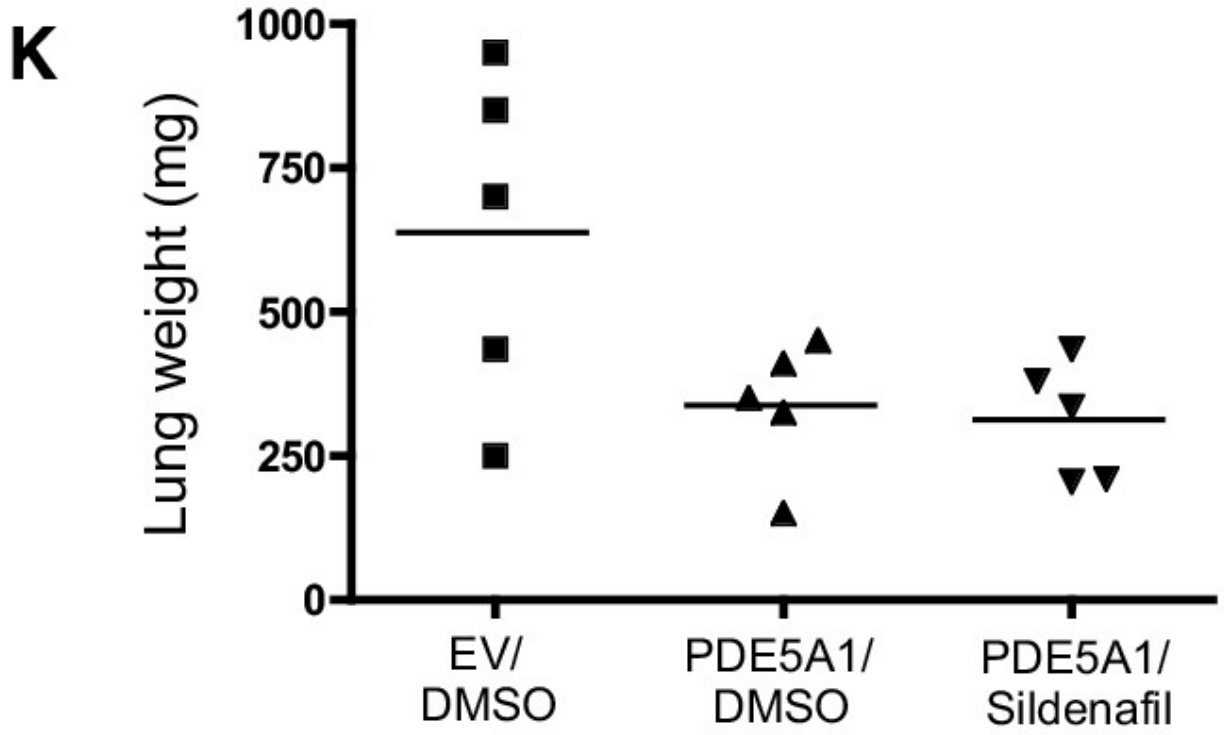




Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011, at p. 50, Fig. 4G.

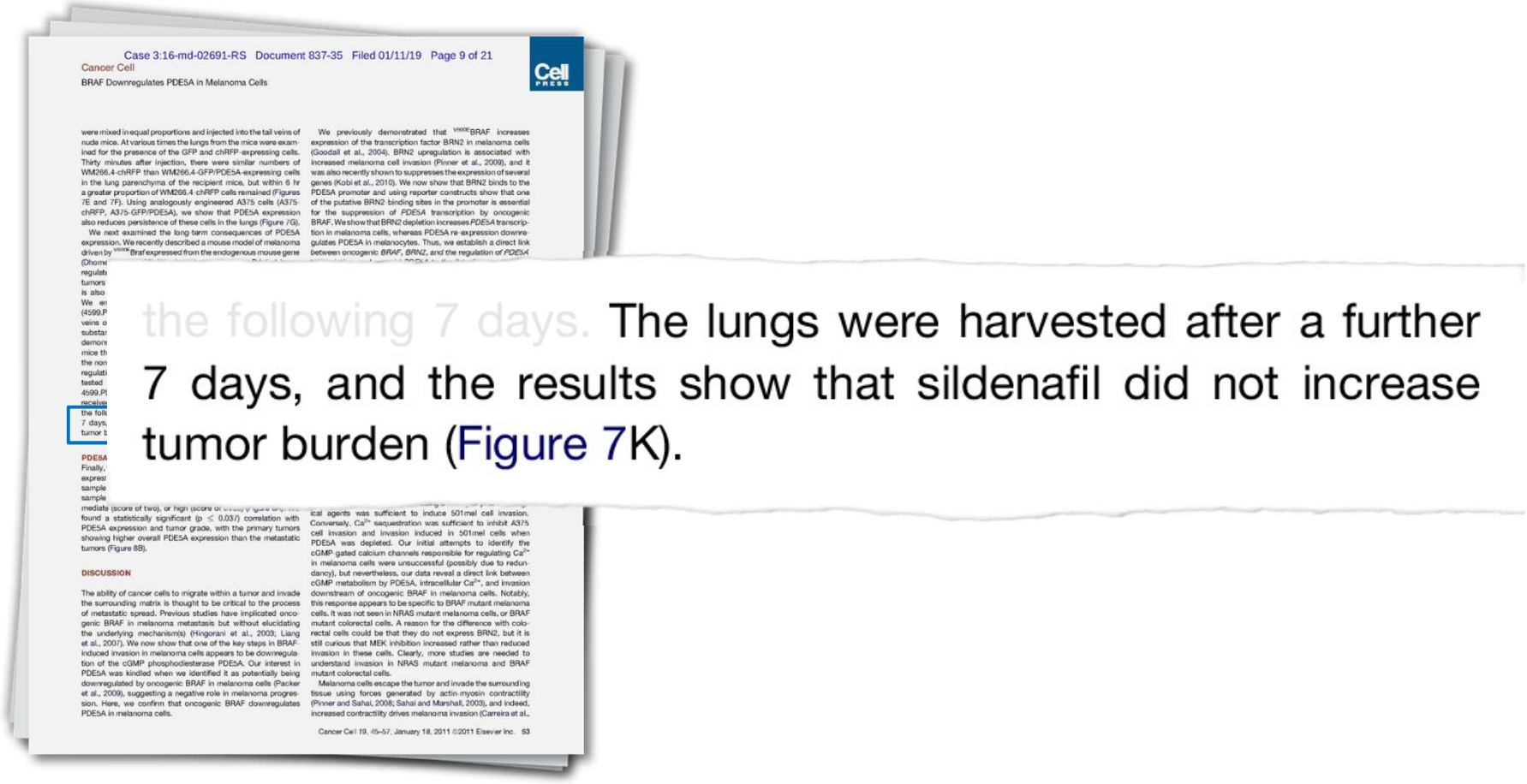
In Vivo: No Increase in Lung Tumor Burden





Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011, at p. 54, Fig. 7K.

In Vivo: No Increase in Lung Tumor Burden



Cancer Cell

BRAF Downregulates PDE5A in Melanoma Cells

Cell PRESS

were mixed in equal proportions and injected into the tail veins of nude mice. At various times the lungs from the mice were examined for the presence of the GFP and chRFP-expressing cells. Thirty minutes after injection, there were similar numbers of WM266.4-chRFP than WM266.4-GFP/PDE5A-expressing cells in the lung parenchyma of the recipient mice, but within 6 hr a greater proportion of WM266.4-chRFP cells remained (Figures 7E and 7F). Using analogously engineered A375 cells (A375-chRFP, A375-GFP/PDE5A), we show that PDE5A expression also reduces persistence of these cells in the lungs (Figure 7G).

We next examined the long-term consequences of PDE5A expression. We recently described a mouse model of melanoma driven by ^{V600E}Braf expressed from the endogenous mouse gene

We previously demonstrated that ^{V600E}BRAF increases expression of the transcription factor BRN2 in melanoma cells (Goodall et al., 2004). BRN2 upregulation is associated with increased melanoma cell invasion (Pinner et al., 2009), and it was also recently shown to suppress the expression of several genes (Kobi et al., 2010). We now show that BRN2 binds to the PDE5A promoter and using reporter constructs show that one of the putative BRN2-binding sites in the promoter is essential for the suppression of *PDE5A* transcription by oncogenic BRAF. We show that BRN2 depletion increases *PDE5A* transcription in melanoma cells, whereas PDE5A re-expression downregulates PDE5A in melanocytes. Thus, we establish a direct link between oncogenic *BRAF*, *BRN2*, and the regulation of *PDE5A*

the following 7 days. The lungs were harvested after a further 7 days, and the results show that sildenafil did not increase tumor burden (Figure 7K).

found a statistically significant ($p < 0.03$) correlation with PDE5A expression and tumor grade, with the primary tumors showing higher overall PDE5A expression than the metastatic tumors (Figure 8B).

DISCUSSION

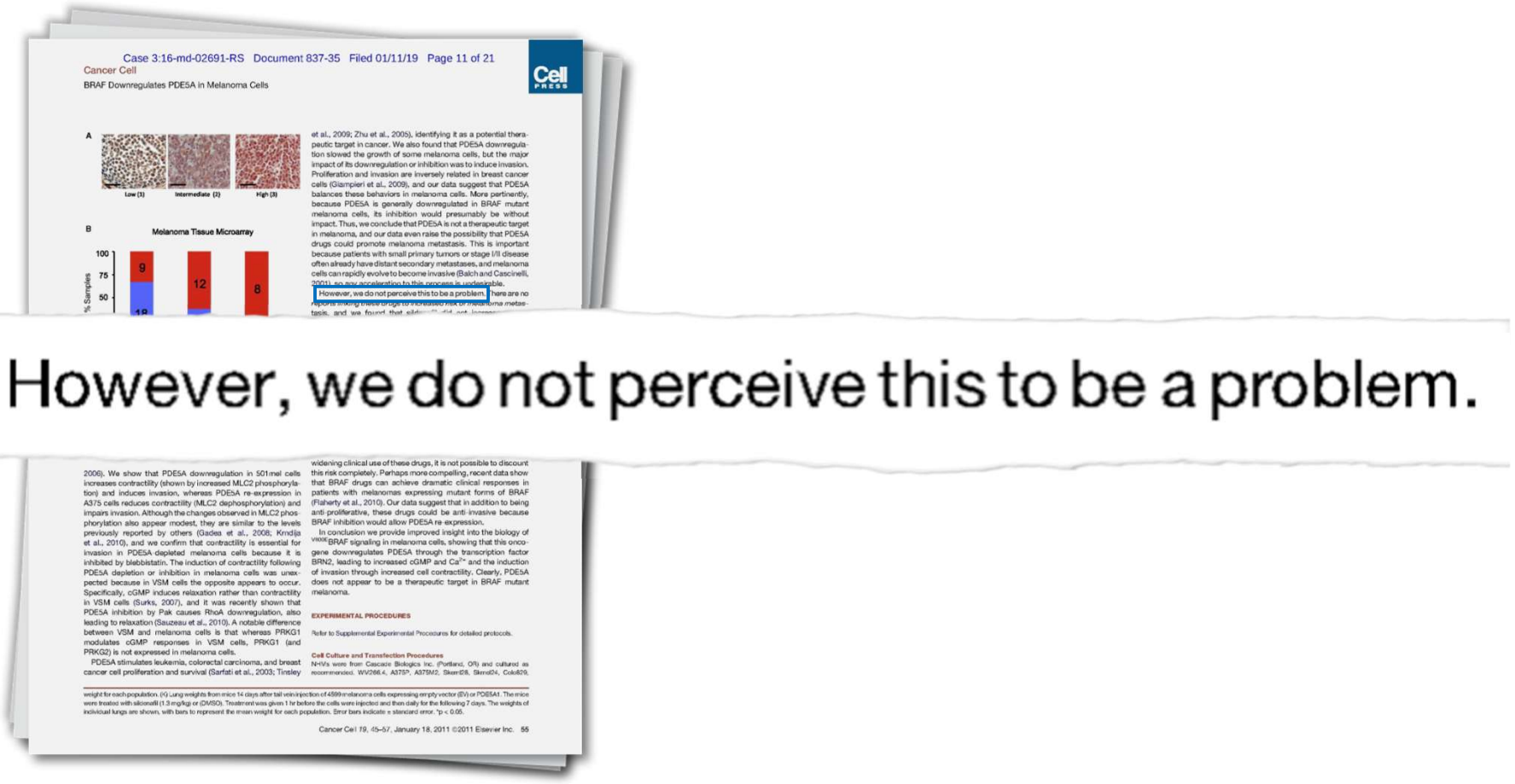
The ability of cancer cells to migrate within a tumor and invade the surrounding matrix is thought to be critical to the process of metastatic spread. Previous studies have implicated oncogenic BRAF in melanoma metastasis but without elucidating the underlying mechanism(s) (Hingorani et al., 2003; Liang et al., 2007). We now show that one of the key steps in BRAF-induced invasion in melanoma cells appears to be downregulation of the cGMP phosphodiesterase PDE5A. Our interest in PDE5A was kindled when we identified it as potentially being downregulated by oncogenic BRAF in melanoma cells (Packer et al., 2009), suggesting a negative role in melanoma progression. Here, we confirm that oncogenic BRAF downregulates PDE5A in melanoma cells.

ical agents was sufficient to induce 501mel cell invasion. Conversely, Ca^{2+} sequestration was sufficient to inhibit A375 cell invasion and invasion induced in 501mel cells when PDE5A was depleted. Our initial attempts to identify the cGMP gated calcium channels responsible for regulating Ca^{2+} in melanoma cells were unsuccessful (possibly due to redundancy), but nevertheless, our data reveal a direct link between cGMP metabolism by PDE5A, intracellular Ca^{2+}, and invasion downstream of oncogenic BRAF in melanoma cells. Notably, this response appears to be specific to BRAF mutant melanoma cells. It was not seen in NRAS mutant melanoma cells, or BRAF mutant colorectal cells. A reason for the difference with colorectal cells could be that they do not express BRN2, but it is still curious that MEK inhibition increased rather than reduced invasion in these cells. Clearly, more studies are needed to understand invasion in NRAS mutant melanoma and BRAF mutant colorectal cells.

Melanoma cells escape the tumor and invade the surrounding tissue using forces generated by actin-myosin contractility (Pinner and Sahai, 2008; Sahai and Marshall, 2003), and indeed, increased contractility drives melanoma invasion (Carreira et al.,

Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011, at p. 53.

Discussion: "We Do Not Perceive [PDE5 Inhibitors] To Be A Problem"



Cancer Cell

BRAF Downregulates PDE5A in Melanoma Cells

Cell PRESS

A

Low (1) Intermediate (2) High (3)

B

Melanoma Tissue Microarray

100 75 50 % Samples 9 12 8

et al., 2009; Zhu et al., 2005), identifying it as a potential therapeutic target in cancer. We also found that PDE5A downregulation slowed the growth of some melanoma cells, but the major impact of its downregulation or inhibition was to induce invasion. Proliferation and invasion are inversely related in breast cancer cells (Giampieri et al., 2009), and our data suggest that PDE5A balances these behaviors in melanoma cells. More pertinently, because PDE5A is generally downregulated in BRAF mutant melanoma cells, its inhibition would presumably be without impact. Thus, we conclude that PDE5A is not a therapeutic target in melanoma, and our data even raise the possibility that PDE5A drugs could promote melanoma metastasis. This is important because patients with small primary tumors or stage I/II disease often already have distant secondary metastases, and melanoma cells can rapidly evolve to become invasive (Balch and Cascinelli, 2001), so any acceleration to this process is undesirable.

However, we do not perceive this to be a problem. There are no

tasis, and we found that

2006). We show that PDE5A downregulation in 501mel cells increases contractility (shown by increased MLC2 phosphorylation) and induces invasion, whereas PDE5A re-expression in A375 cells reduces contractility (MLC2 dephosphorylation) and impairs invasion. Although the changes observed in MLC2 phosphorylation also appear modest, they are similar to the levels previously reported by others (Gadea et al., 2008; Kmdja et al., 2010), and we confirm that contractility is essential for invasion in PDE5A depleted melanoma cells because it is inhibited by blebbistatin. The induction of contractility following PDE5A depletion or inhibition in melanoma cells was unexpected because in VSM cells the opposite appears to occur. Specifically, cGMP induces relaxation rather than contractility in VSM cells (Surks, 2007), and it was recently shown that PDE5A inhibition by Pak causes RhoA downregulation, also leading to relaxation (Sauzeau et al., 2010). A notable difference between VSM and melanoma cells is that whereas PRKG1 modulates cGMP responses in VSM cells, PRKG1 (and PRKG2) is not expressed in melanoma cells.

PDE5A stimulates leukemia, colorectal carcinoma, and breast cancer cell proliferation and survival (Sarfati et al., 2003; Tinsley

widening clinical use of these drugs, it is not possible to discount this risk completely. Perhaps more compelling, recent data show that BRAF drugs can achieve dramatic clinical responses in patients with melanomas expressing mutant forms of BRAF (Flaherty et al., 2010). Our data suggest that in addition to being anti-proliferative, these drugs could be anti-invasive because BRAF inhibition would allow PDE5A re-expression.

In conclusion we provide improved insight into the biology of ^{V600E}BRAF signaling in melanoma cells, showing that this oncogene downregulates PDE5A through the transcription factor BRN2, leading to increased cGMP and Ca^{2+} and the induction of invasion through increased cell contractility. Clearly, PDE5A does not appear to be a therapeutic target in BRAF mutant melanoma.

EXPERIMENTAL PROCEDURES

Refer to Supplemental Experimental Procedures for detailed protocols.

Cell Culture and Transfection Procedures

NHAs were from Cascade Biologics Inc. (Portland, OR) and cultured as recommended. WV266.4, A375P, A375M2, Skmel28, Skmel24, Colo829,

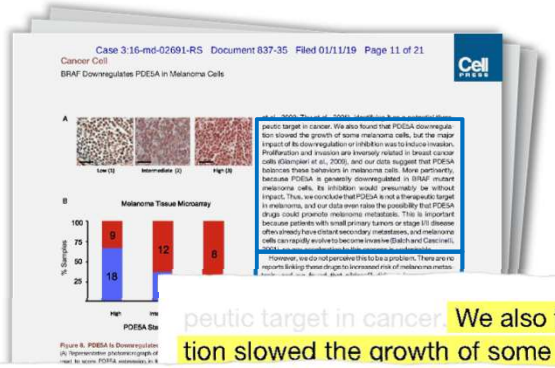
weight for each population. (H) Lung weights from mice 14 days after tail vein injection of 4599 melanoma cells expressing empty vector (EV) or PDE5A1. The mice were treated with sildenafil (1.3 mg/kg) or (DMSO). Treatment was given 1 hr before the cells were injected and then daily for the following 7 days. The weights of individual lungs are shown, with bars to represent the mean weight for each population. Error bars indicate ± standard error. *p < 0.05.

Cancer Cell 19, 45–57, January 18, 2011 ©2011 Elsevier Inc. 55

However, we do not perceive this to be a problem.

Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011, at p. 55.

Discussion: "We Do Not Perceive [PDE5 Inhibitors] To Be A Problem"



peutic target in cancer. We also found that PDE5A downregulation slowed the growth of some melanoma cells, but the major impact of its downregulation or inhibition was to induce invasion. because PDE5A is generally downregulated in BRAF mutant melanoma cells, its inhibition would presumably be without impact. Thus, we conclude that PDE5A is not a therapeutic target cells can rapidly evolve to become invasive (Balch and Cascine in melanoma, and our data even raise the possibility that PDE5A drugs could promote melanoma metastasis. This is important

However, we do not perceive this to be a problem. There are no reports linking these drugs to increased risk of melanoma metastasis, and we found that sildenafil did not increase mouse

1

tasis, and we found that sildenafil did not increase mouse lung colonization by melanoma cells. Furthermore, PDE5A drugs

2

lung colonization by melanoma cells. Furthermore, PDE5A drugs are generally used as needed rather than persistently and are generally cleared rapidly ($T_{1/2}$ ~2 hr) because their effects must be short lived. Moreover, in addition to being able to degrade

3

cGMP, phosphodiesterases appear to possess enzyme-independent functions, as implied by their interaction with many other cellular proteins (Houslay, 2010). Thus, we posit that complete

4

cellular proteins (Houslay, 2010). Thus, we posit that complete loss of PDE5A protein is not akin to its transient and reversible inhibition that is mediated by drugs. Furthermore, as mentioned,

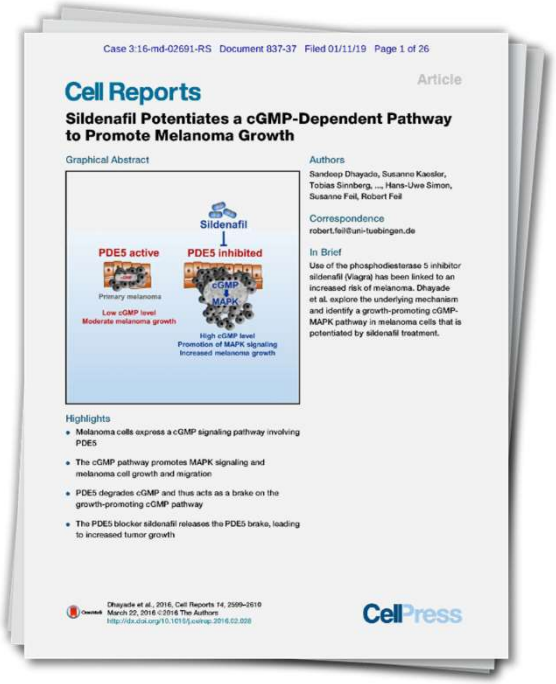
5

because PDE5A is already downregulated in most BRAF mutant melanoma cases, its further inhibition is presumably not possible.

6

Source: JX 85, Arozarena et al., Cancer Cell 19, 45-57, Jan. 18, 2011, at p. 55.

Why Plaintiffs' Experts Unreliably Interpret Dhayade et al.



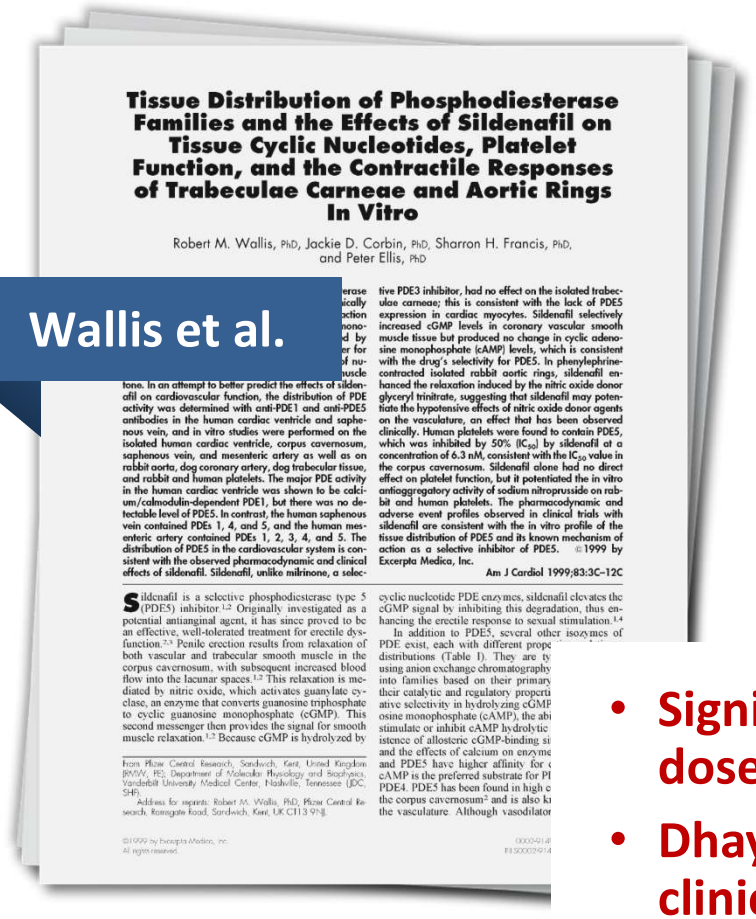
1 The extremely high doses of sildenafil used by Dhayade have off-target effects

- Plaintiffs' experts rely on cGMP levels in mouse hearts
  - But there is no detectable PDE5 in the heart
  - Other PDEs [e.g., PDE1] are present in the heart
- In the Dhayade study, there were very low (non-functional) levels of PDE5 in melanoma cells, but PDE1 was present at high levels
- Therefore, the effects Dhayade observed must be due to off-target effects of their very high doses

2 Plaintiffs' experts ignore inconsistent growth results in Dhayade and Zhang

Source: JX 87, Dhayade et al., Cell Reports 14, 2599-2610, Mar. 22, 2016.

Extremely High Doses of Sildenafil Inhibit PDE1



Tissue Distribution of Phosphodiesterase Families and the Effects of Sildenafil on Tissue Cyclic Nucleotides, Platelet Function, and the Contractile Responses of Trabeculae Carneae and Aortic Rings In Vitro

Robert M. Wallis, PhD, Jackie D. Corbin, PhD, Sharron H. Francis, PhD, and Peter Ellis, PhD

Wallis et al.

Family	Geometric Mean IC_{50} (nM)
PDE1	280
PDE5	3.5

- **Significantly exceeding the maximum clinical dose will inhibit <u>both</u> PDE1 and PDE5**
- **Dhayade used more than <u>180x</u> the maximum clinical dose – more than enough to inhibit PDE1**

Source: DX 132, Wallis et al., Am. J. Cardiology 1999;83:3C-12C, at 4C (Table 1), 6C.

Dhayade Used Extremely High Doses of Sildenafil

Human Dose

1.25 mg/kg

(one 100 mg pill per week)

Arozarena

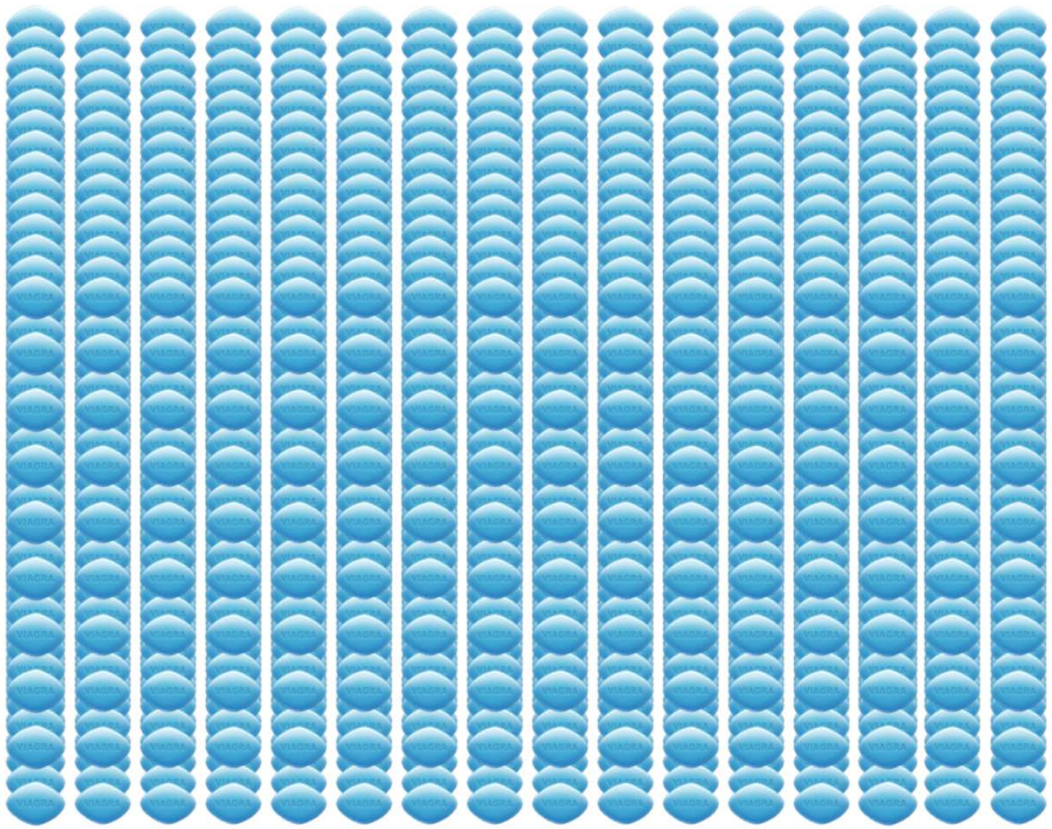
1.3 mg/kg

(every day for 7 days)

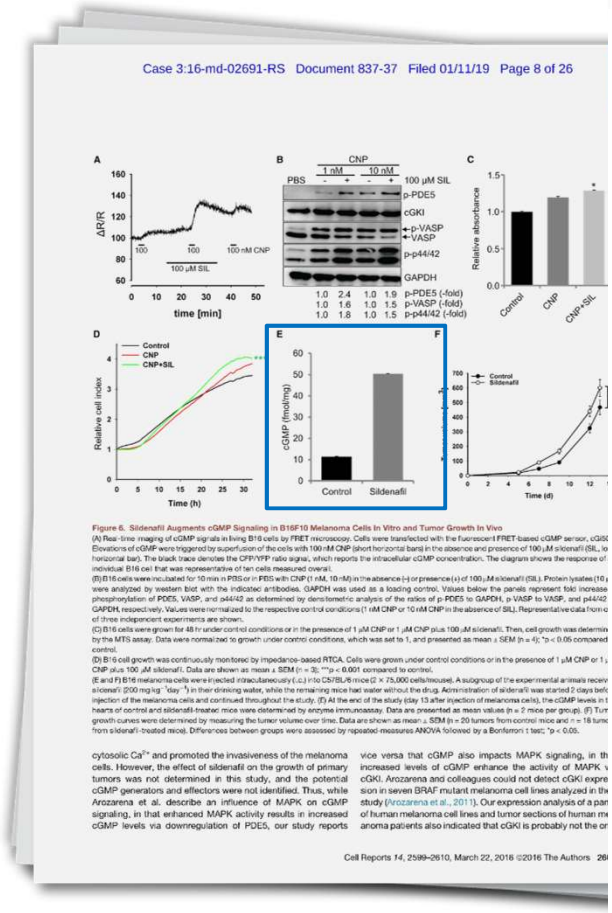
Dhayade

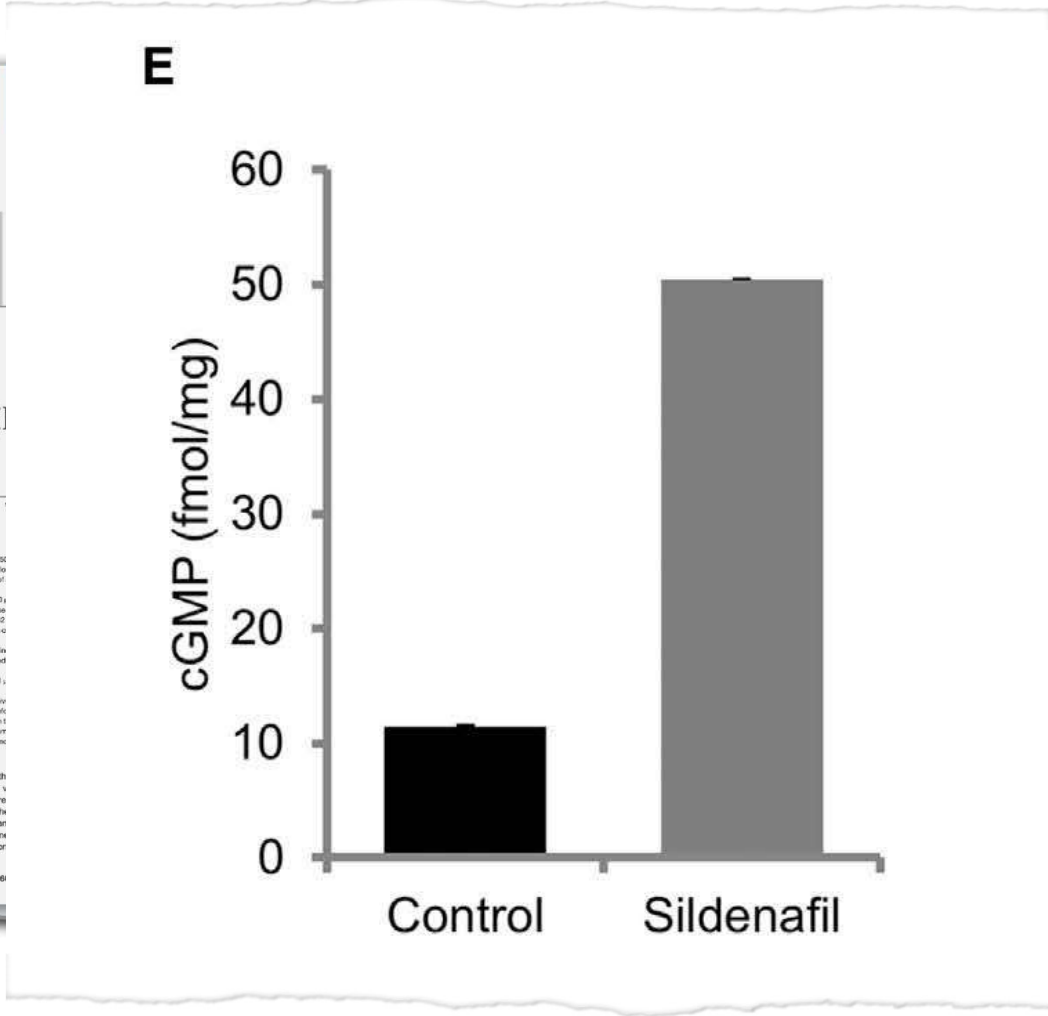
200 mg/kg

(every day for 14 days)



Dhayade: Plaintiffs' Experts Rely on cGMP Levels in Hearts of Mice





Source: JX 87, Dhayade et al., Cell Reports 14, 2599-2610, Mar. 22, 2016, at p. 2605, Fig. 6E.

Virtually No PDE5 in the Heart – But PDE1 Is Abundant

Degen et al.

Our data clearly demonstrate that we are unable to detect PDE5 in any of the cardiac tissue lysates examined from humans or experimental models of HF, whereas PDE5 is present in the murine and bovine lung samples used as a positive control. These results indicate that if PDE5

Wallis et al.

Family	Tissue	Tissue Localization
PDE1	Cardiac ventricle	Brain, heart, kidney, liver, skeletal muscle, vascular and visceral smooth muscle
PDE5	Corpus cavernosum	Corpus cavernosum, platelets, skeletal muscle, vascular and visceral smooth muscle

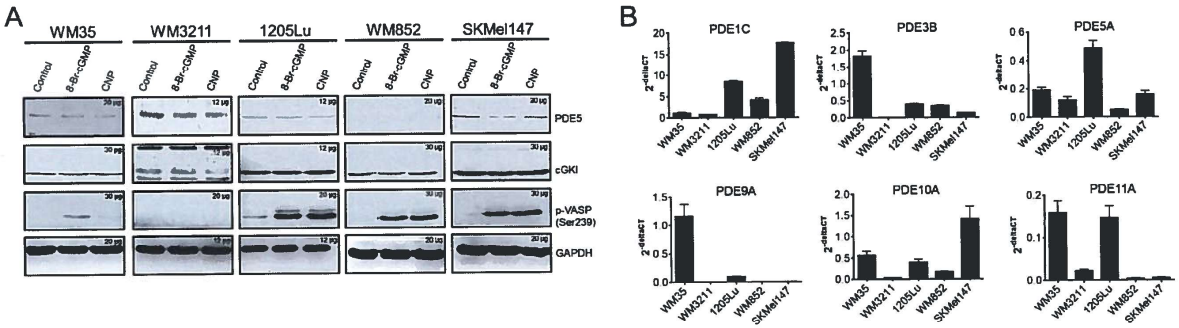
The change in cGMP levels observed in Dhayade in the heart cannot be explained by inhibition of PDE5 – it has to be other PDEs (such as PDE1)

Sources: DX 110, Degen, et al., The Emperor's New Clothes: PDE5 and the Heart, PLoS One. 2015 Mar 6; 10(3):e0118664; DX 132, Wallis et al., Am. J. Cardiology 1999;83:3C-12C, at 4C (Table 1), 6C.

Haq Slide 57 (10/15/2019)

ANALYSIS OF PDE5IS AND MELANOMA

PDE5 is present in many melanomas cells



Dhayade (2016), Fig. S2 – JX087

57

Source: Haq Slide 57 (10/15/2019).

PDE5 Is Barely Present in Melanomas – But PDE1 Is

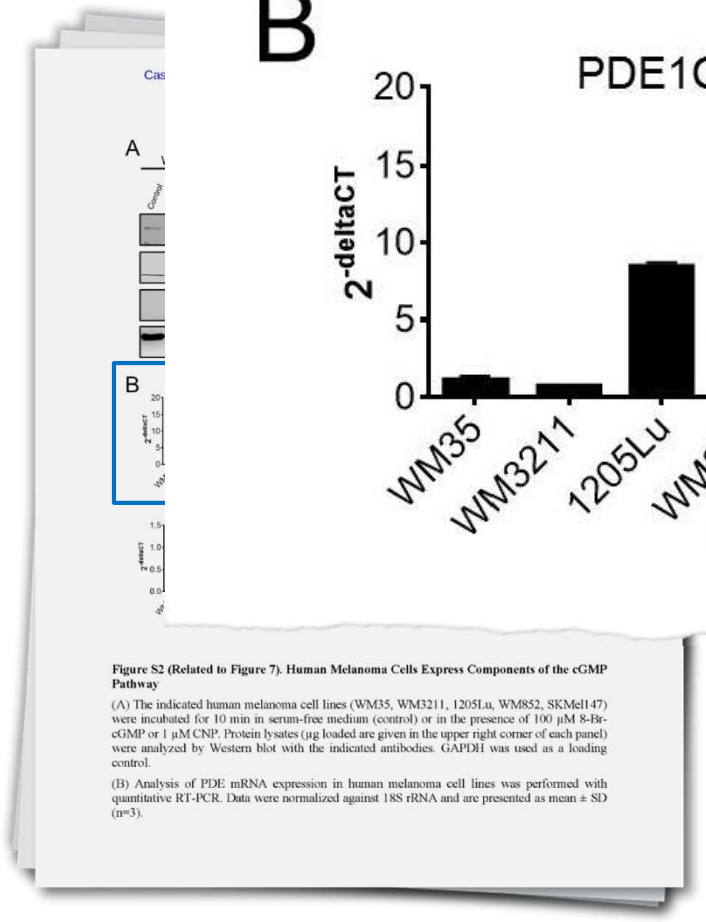
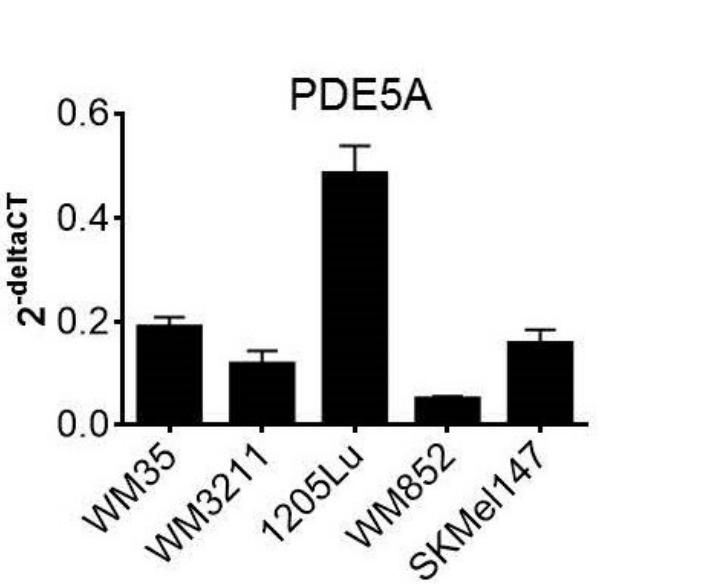


Figure S2 (Related to Figure 7). Human Melanoma Cells Express Components of the cGMP Pathway

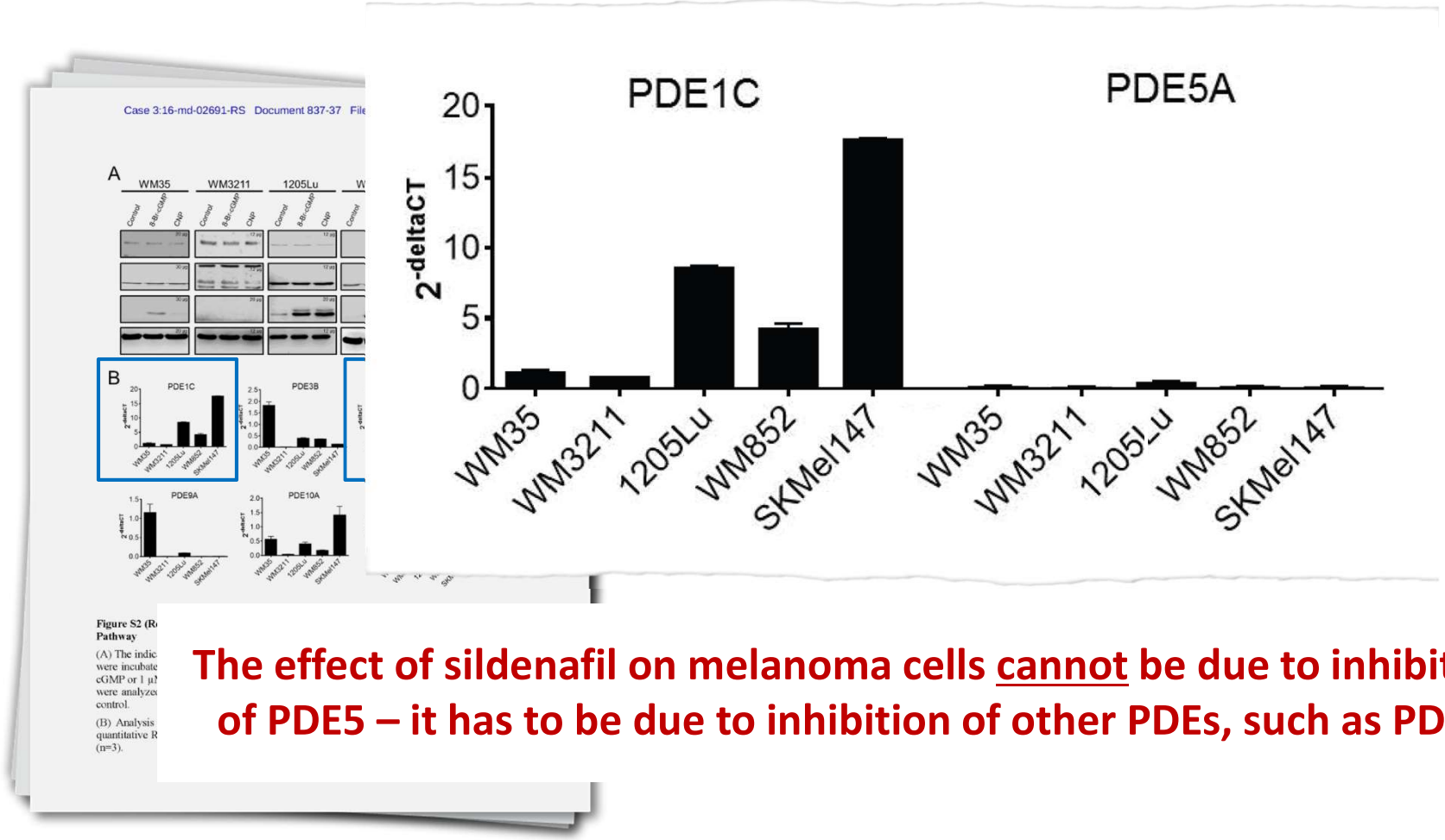
(A) The indicated human melanoma cell lines (WM35, WM3211, 1205Lu, WM852, SKMel147) were incubated for 10 min in serum-free medium (control) or in the presence of 100 μM 8-Br-cGMP or 1 μM CNP. Protein lysates (μg loaded are given in the upper right corner of each panel) were analyzed by Western blot with the indicated antibodies. GAPDH was used as a loading control.

(B) Analysis of PDE mRNA expression in human melanoma cell lines was performed with quantitative RT-PCR. Data were normalized against 18S rRNA and are presented as mean ± SD (n=3).



Source: JX 87, Dhayade et al., Cell Reports 14, 2599-2610, Mar. 22, 2016, at supplement, Fig. S2B.

PDE5 Is Barely Present in Melanomas – But PDE1 Is

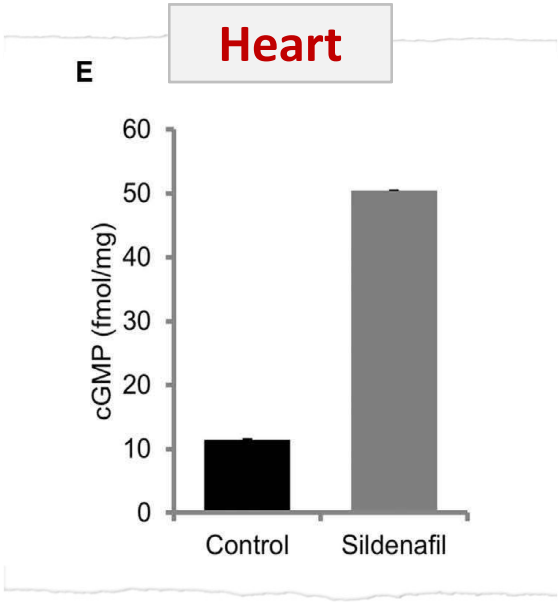


The effect of sildenafil on melanoma cells cannot be due to inhibition of PDE5 – it has to be due to inhibition of other PDEs, such as PDE1

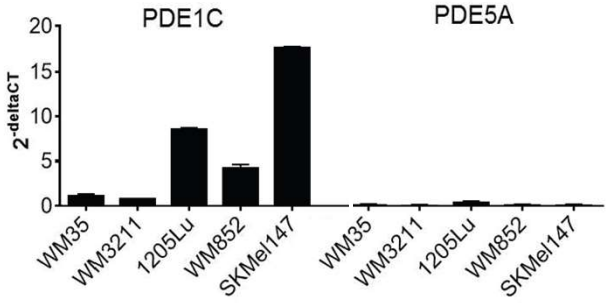
Source: JX 87, Dhayade et al., Cell Reports 14, 2599-2610, Mar. 22, 2016, at supplement, Fig. S2B.

Plaintiffs' Experts Ignore Off-Target Effects of High Dose on Other PDEs

Heart



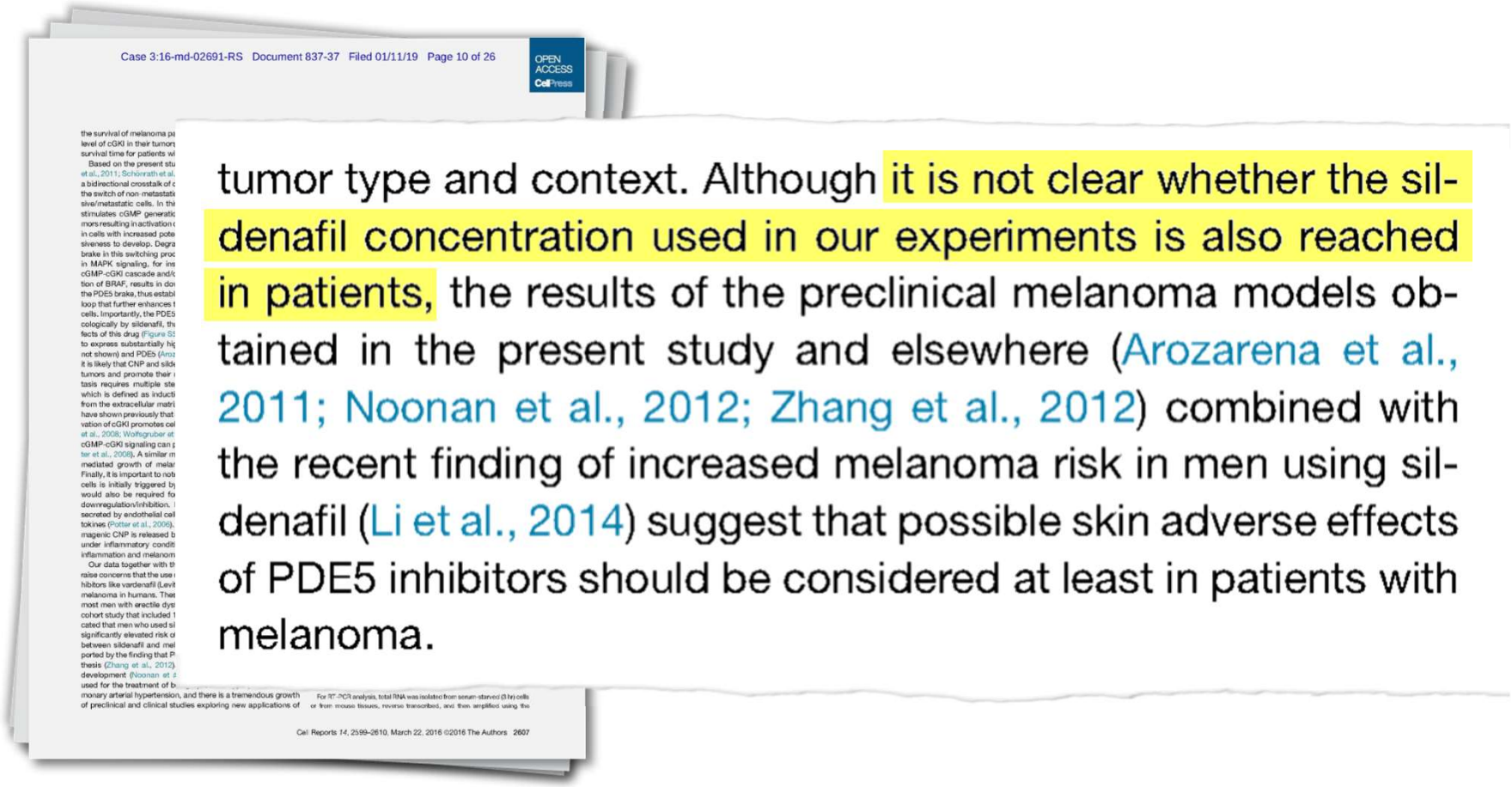
Melanoma



- **The change in cGMP levels in the heart cannot be explained by inhibition of PDE5 – it has to be other PDEs (such as PDE1)**

- **The effects of sildenafil in melanoma cells cannot be explained by inhibition of PDE5 – it has to be other PDEs (such as PDE1)**

Dhayade Admits Their Dose May Not Be Reached in Patients

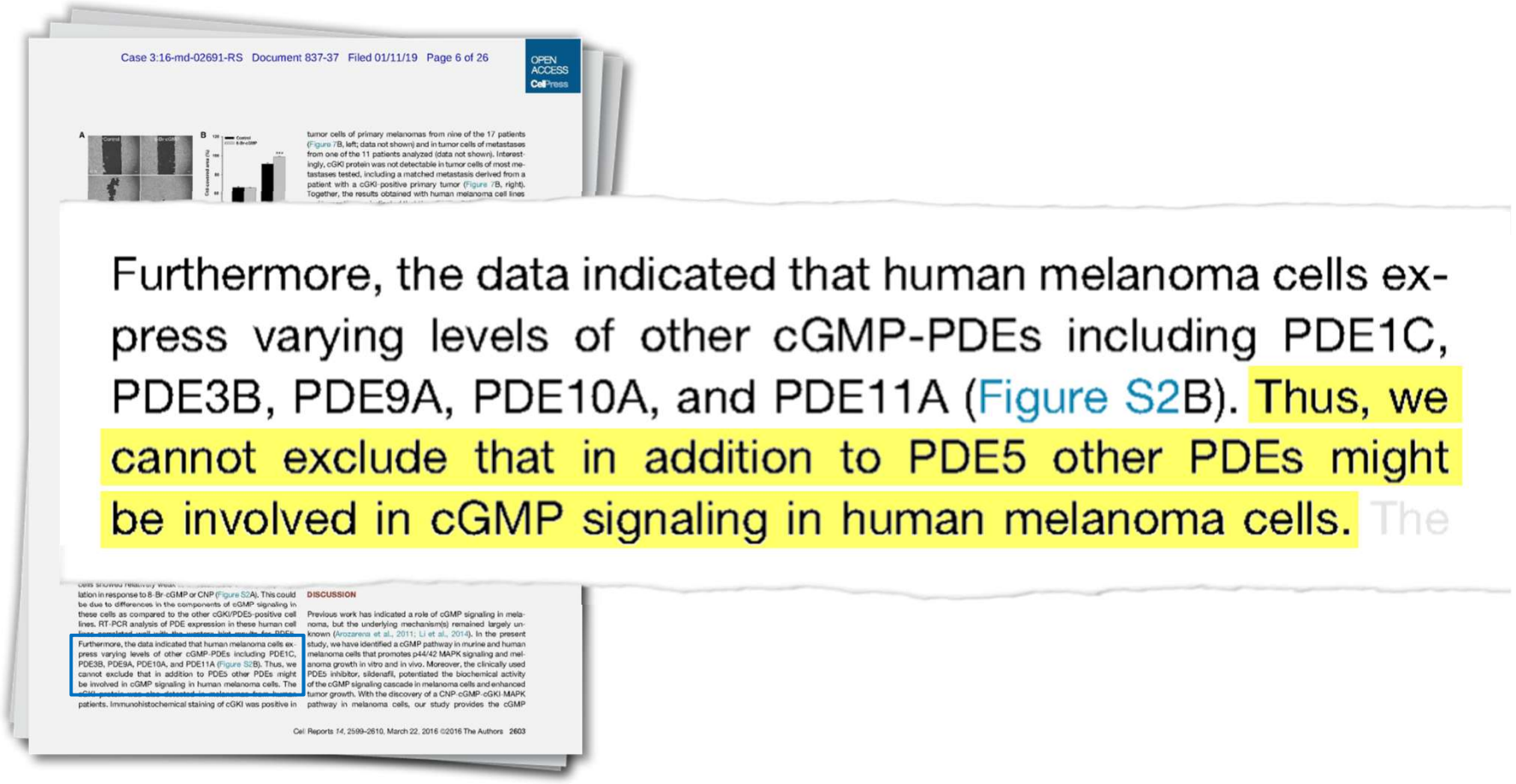


OPEN ACCESS CellPress

tumor type and context. Although it is not clear whether the sildenafil concentration used in our experiments is also reached in patients, the results of the preclinical melanoma models obtained in the present study and elsewhere (Arozarena et al., 2011; Noonan et al., 2012; Zhang et al., 2012) combined with the recent finding of increased melanoma risk in men using sildenafil (Li et al., 2014) suggest that possible skin adverse effects of PDE5 inhibitors should be considered at least in patients with melanoma.

Source: JX 87, Dhayade et al., Cell Reports 14, 2599-2610, Mar. 22, 2016, at p. 2607.

Dhayade Admits Other PDEs Play Role in cGMP Signaling



OPEN ACCESS CellPress

tumor cells of primary melanomas from nine of the 17 patients (Figure 7B, left; data not shown) and in tumor cells of metastases from one of the 11 patients analyzed (data not shown). Interestingly, cGKI protein was not detectable in tumor cells of most metastases tested, including a matched metastasis derived from a patient with a cGKI-positive primary tumor (Figure 7B, right). Together, the results obtained with human melanoma cell lines

Furthermore, the data indicated that human melanoma cells express varying levels of other cGMP-PDEs including PDE1C, PDE3B, PDE9A, PDE10A, and PDE11A (Figure S2B). Thus, we cannot exclude that in addition to PDE5 other PDEs might be involved in cGMP signaling in human melanoma cells. The

lation in response to 8-Br-cGMP or CNP (Figure S2A). This could be due to differences in the components of cGMP signaling in these cells as compared to the other cGKI/PDE5-positive cell lines. RT-PCR analysis of PDE expression in these human cell

Furthermore, the data indicated that human melanoma cells express varying levels of other cGMP-PDEs including PDE1C, PDE3B, PDE9A, PDE10A, and PDE11A (Figure S2B). Thus, we cannot exclude that in addition to PDE5 other PDEs might be involved in cGMP signaling in human melanoma cells. The

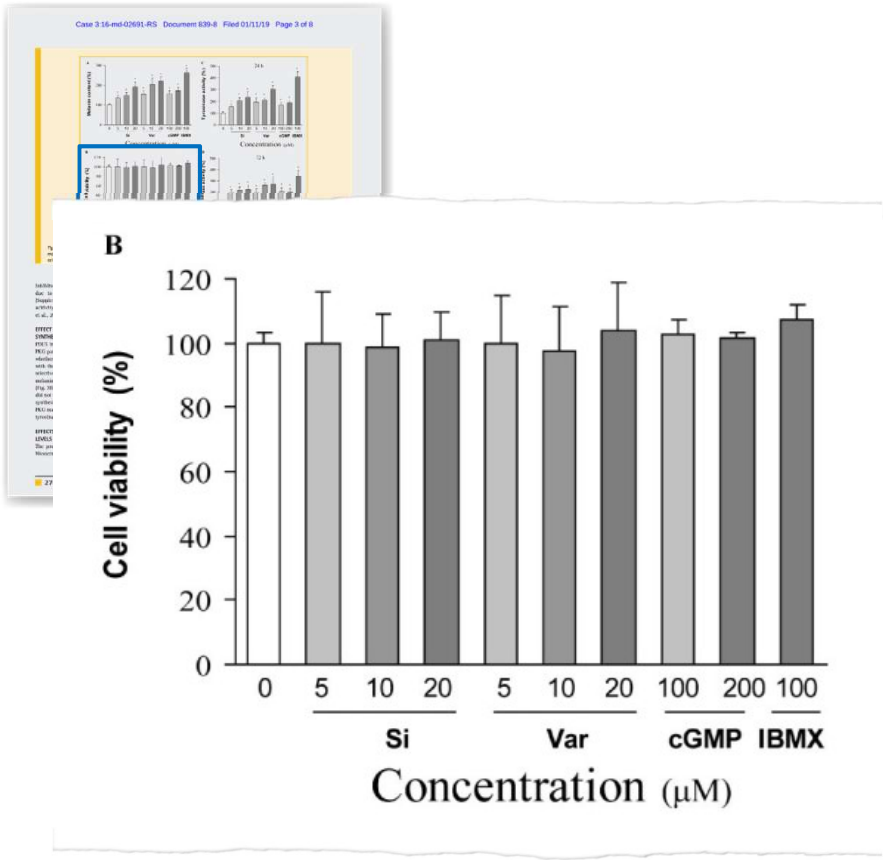
patients. Immunohistochemical staining of cGKI was positive in

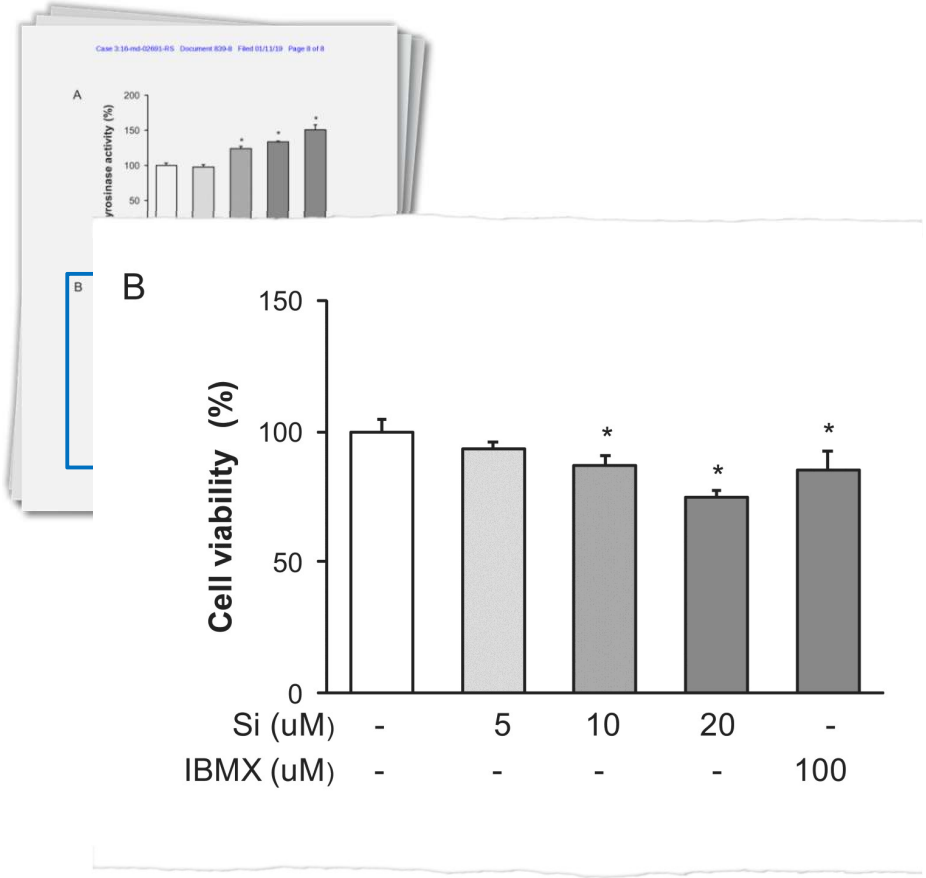
DISCUSSION

Previous work has indicated a role of cGMP signaling in melanoma, but the underlying mechanism(s) remained largely unknown (Arozarena et al., 2011; Li et al., 2014). In the present study, we have identified a cGMP pathway in murine and human melanoma cells that promotes p44/42 MAPK signaling and melanoma growth in vitro and in vivo. Moreover, the clinically used PDE5 inhibitor, sildenafil, potentiated the biochemical activity of the cGMP signaling cascade in melanoma cells and enhanced tumor growth. With the discovery of a CNP-cGMP-cGKI-MAPK pathway in melanoma cells, our study provides the cGMP

Source: JX 87, Dhayade et al., Cell Reports 14, 2599-2610, Mar. 22, 2016, at p. 2603.

Zhang: Did Not Replicate Dhayade Growth Results





Mouse Melanoma Cells:
No Significant Change in Growth

Human Melanoma Cells:
Significant Decrease in Growth

Source: JX 118, Zhang et al., J. Cell. Biochem, Mar.15 2012, at p. 2740, Fig. 1B, supplement, Fig. S1B.

Totality of Evidence Does Not Establish Biological Plausibility

Study	Growth with PDE5 Inhibitors?	Invasion with PDE5 Inhibitors?
 Arozarena	**NO**	*In vitro*: 1 cell line *In vivo*: **NO**
 Dhayade	**YES**	**NO**
 Zhang	**NO**	**NO**